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## Microbial Transformation of a Series of Androgens with *Aspergillus tamarii*

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Incubation of androsterone (5) with *Aspergillus tamarii* gave  $11\alpha$  and  $11\beta$  hydroxylation of 5. Isoandrosterone was converted into  $11\beta$ -hydroxyisoandrosterone. Incubation of 1,4-androstadiene-3,17-dione (12) gave  $11\alpha$  and  $11\beta$  hydroxylation of 12, 1,4-androstadien-17 $\beta$ -ol-3-one (13), and 1,4-androstadiene- $11\beta$ ,17 $\beta$ -diol-3-one (14). Compound 13 was shown to be the precursor of 14. Adrenosterone and 4-androstene-3,11,17-trione gave the respective 17 $\beta$ -hydroxy derivatives. Incubation of 5 $\beta$ -androstan-3,17-dione gave 5 $\beta$ -androstan-7 $\beta$ -ol-3,17-dione, whereas 5 $\alpha$ -androstan-3,17-dione (24) gave 5 $\alpha$ -androstan-6 $\beta$ -ol-3,17-dione and  $11\beta$ -hydroxy-5 $\alpha$ -dihydrotestololactone (27). Androstanolone gave 5 $\alpha$ -androstan- $11\beta$ ,17 $\beta$ -diol-3-one (30), 24, and 27. Compound 30 was shown not to be a precursor of 27. Analogously with the progesterone to testololactone conversion, 5 $\alpha$ -pregnane-3,20-dione was converted into 5 $\alpha$ -dihydrotestololactone (32) and 27. A C-11 hydroxyl substituent has been shown to inhibit transformations by *A. tamarii* which do occur with the unsubstituted androgens. A discussion of microbial hydroxylation substrate specificity is presented.

The sequential removal of the side chain from C<sub>21</sub> steroids by microorganisms to yield D-ring lactones was first reported by Peterson, *et al.*,<sup>1</sup> and by Fried and co-workers.<sup>2</sup> Since then numerous organisms have been found<sup>3-8</sup> which will remove an acetyl,  $\alpha$ -ketol, or dihydroxyacetone side chain from pregnene steroids to yield testololactone or its derivatives.

More than one mechanism has been shown<sup>9,10</sup> to be operative in the microbial removal of the acetyl

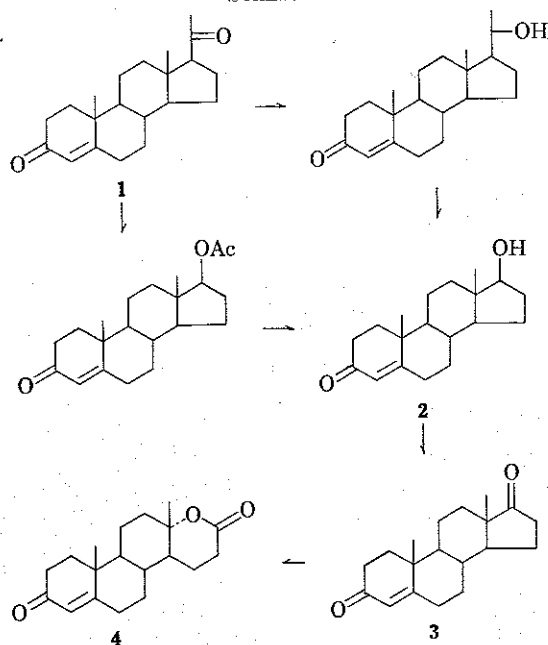
side chain of progesterone (1) as shown in Scheme I. However, insertion of the ethereal oxygen between C-13 and C-17 of 3 is in accordance with the chemical mechanism of per acid attack on the C-17 ketone to produce the lactone as shown in Scheme I.<sup>2,11</sup>

In an earlier study<sup>12</sup> incubation of progesterone (1) with *Aspergillus tamarii* Kita gave testololactone (4) as the major metabolite. A second product from incubation of this substrate was  $11\beta$ -hydroxytestosterone, which appeared to be formed from hydroxylation of testosterone (2). Although 2 is an intermediate in the conversion of progesterone to testololactone, the  $11\beta$ -hydroxy analog of 2 is not metabolized further by this fungus. This inability of *A. tamarii* to metabolize

- (1) D. H. Peterson, *et al.*, *J. Am. Chem. Soc.*, **75**, 5768 (1953).
- (2) J. Fried, R. W. Thoma, and A. Klingsberg, *ibid.*, **75**, 5764 (1953).
- (3) A. Čapek, *et al.*, *Naturwissenschaften*, **43**, 471 (1956).
- (4) A. Bodanszky, J. Kollonitsch, and A. Wix, *Experientia*, **11**, 384 (1955).
- (5) M. Nishikawa, S. Noguchi, and T. Hasegawa, *Pharm. Bull. (Tokyo)*, **3**, 322 (1955).
- (6) G. E. Peterson, *et al.*, *J. Bacteriol.*, **74**, 684 (1957).
- (7) A. Čapek and O. Hanč, *Folia Microbiol. (Prague)*, **5**, 251 (1960).
- (8) A. Wintraub, S. H. Eppstein, and P. D. Meister, German Patent 1,021,845 (Jan 2, 1958); *Chem. Abstr.*, **54**, 4686 (1958).
- (9) G. S. Fonken, H. C. Murray, and L. M. Reineke, *J. Am. Chem. Soc.*, **82**, 5507 (1960).

- (10) O. K. Sebek, L. M. Reineke, and D. H. Peterson, *J. Bacteriol.*, **83**, 1327 (1962).
- (11) H. Levy and R. P. Jacobsen, *J. Biol. Chem.*, **171**, 71 (1947).
- (12) D. R. Brannon, *et al.*, *J. Org. Chem.*, **30**, 760 (1965).

SCHEME I



11-hydroxylated androgens was again demonstrated when 11 $\alpha$ -hydroxyandrostendione was recovered unchanged after incubation; 11 $\alpha$ - and 11 $\beta$ -hydroxyprogesterone gave only the corresponding 11-hydroxytestosterone. Incubation of 2 with *A. tamaritii* gave 4 and 11 $\beta$ -hydroxytestosterone, whereas incubation of androstendione (3) gave only 4.

To determine the extent of this apparent competition between the 11-hydroxylase and D-ring lactonase systems of *A. tamaritii*, and in the hope of finding other cases of side-chain cleavage with D-ring lactone formation, shaken cultures of *A. tamaritii* were inoculated with a series of androgens which differ in the A ring.

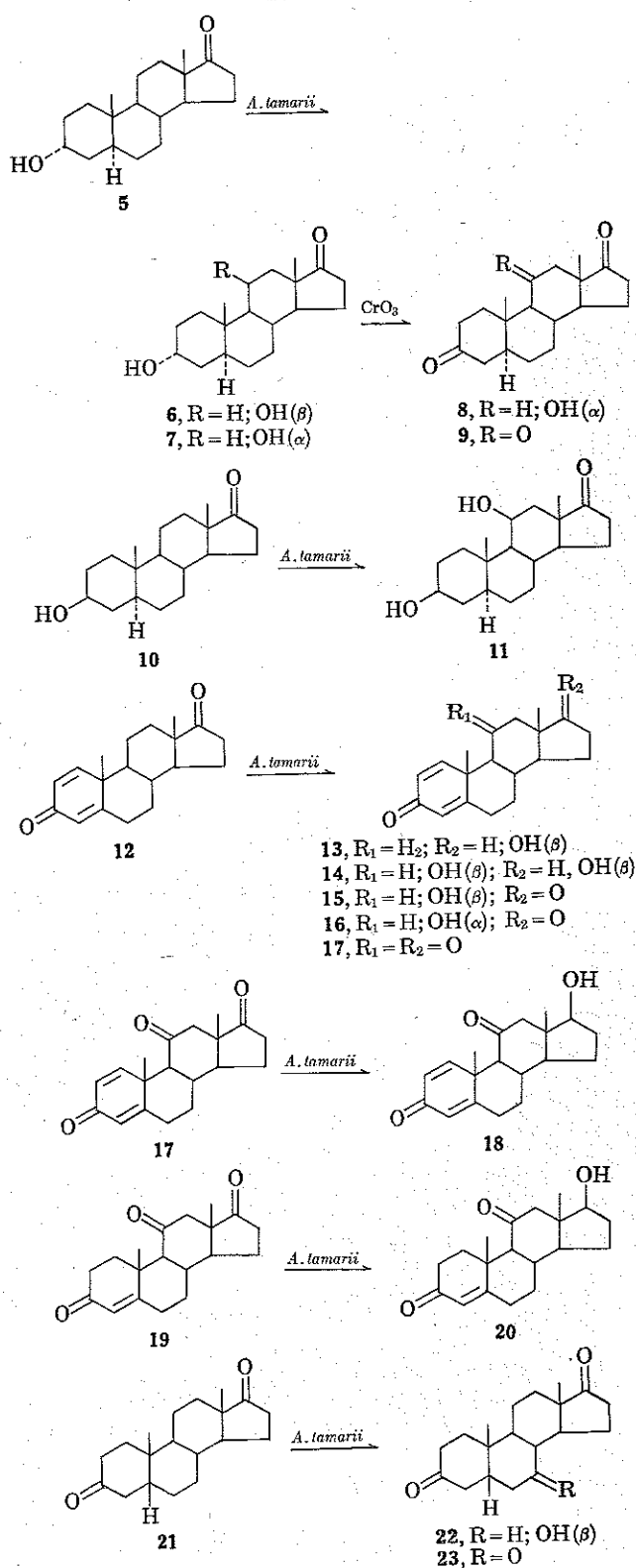
Incubation of *A. tamaritii* with androsterone (5) gave 11 $\beta$ -hydroxyandrosterone (6) as the major metabolite, and a small amount of 7 (Scheme II). The infrared spectrum of 7 showed a hydroxyl absorption and a carbonyl band indicative of either a five-membered cyclic ketone or a six-membered lactone. Mass spectral analysis of 7 gave a molecular weight of 306.2177, which is in agreement with the elemental analysis of compound 7 for  $C_{19}H_{30}O_3$ . These results indicated that the substrate had gained one oxygen atom, either by hydroxylation or lactonization. However, the physical properties of 7 differ from those reported<sup>11</sup> for the corresponding D ring lactone, andrololactone. Oxidation of 7 gave 5 $\alpha$ -androstan-11 $\alpha$ -ol-3,17-dione (8), and 5 $\alpha$ -androstan-3,11,17-trione (9). Based upon this evidence we propose that 7 is 11 $\alpha$ -hydroxyandrosterone, which, to our knowledge, has not been described previously in the literature.

Incubation of isoandrosterone (10) with *A. tamaritii* gave only 11 $\beta$ -hydroxyisoandrosterone (11) as the major metabolite; no 11 $\alpha$ -hydroxyisoandrosterone was isolated.<sup>13</sup>

Incubation of 1,4-androstadiene-3,17-dione (12) for 96 hr gave a product which showed a minor component of  $R_f$  0.75, and three major components of  $R_f$  0.80, 0.60, and 0.65 on examination by tlc (methanol-ether, 1:10). Incubation of 12 for 48 hr gave a mixture

(13) In every case examined tlc of C-11 hydroxyl epimeric pairs showed the  $\alpha$  epimer to have a smaller  $R_f$  value than the  $\beta$  epimer.

SCHEME II



which contained only a trace of the compound of  $R_f$  0.60, but relatively large amounts of the compounds of  $R_f$  0.80, 0.75, and 0.65. The premise that the compound of  $R_f$  0.75 was the precursor of the compound of  $R_f$  0.60 was verified when the compounds of  $R_f$  0.75 and 0.60 were shown to be 1,4-androstadiene-17 $\beta$ -ol-3-one (13) and the corresponding hydroxylated compound, 1,4-androstadiene-11 $\beta$ ,17 $\beta$ -diol-3-one (14), respectively. The product of  $R_f$  0.80 was shown to be 1,4-androsta-

dien-11 $\beta$ -ol-3,17-dione (15); the product of  $R_f$  0.65 exhibited physical properties identical with those of 1,4-androstadien-11 $\alpha$ -ol-3,17-dione (16). Oxidation of 16 gave 1,4-androstadiene-3,11,17-trione (17). Incubation of alcohol 13 produced diol 14, but none of compound 15, nor could 15 be obtained by incubation of diol 14. These results again demonstrate the inability of *A. tamarii* to metabolize 11-hydroxylated androgens.

Talalay<sup>14</sup> has suggested that the formation of epimeric alcohols by microbial hydroxylation, as with androsterone and 1,4-androstadiene-3,17-dione described above, may be the consequence of a single hydroxylase acting in concert with two stereospecific hydroxy steroid dehydrogenases which cause inversion of configuration *via* a ketonic intermediate. However, incubation of 1,4-androstadiene-3,11,17-trione (17) and adrenosterone (19) resulted only in reduction of the respective C-17 ketone to give 1,4-androstadien-17 $\beta$ -ol-3,11-dione (18) and 4-androsten-17 $\beta$ -ol-3,11-dione (20) (see Scheme II).

When 5 $\beta$ -androstane-3,17-dione (21) was incubated with *A. tamarii*, the major metabolite was compound 22. The infrared spectrum of 22 showed a hydroxyl absorption and absorptions owing to five- and six-membered cyclic ketones; however, the physical properties of 22 greatly differ from those of either the 11 $\alpha$ - or 11 $\beta$ -hydroxy derivative of dione 21. Oxidation of compound 22 produced trione 23, whose infrared spectrum was indistinguishable from that of 5 $\beta$ -androstane-3,7,17-trione. Comparison of the physical properties of 22 with those of 5 $\beta$ -androstane-7 $\beta$ -ol-3,17-dione, obtained by incubation<sup>15</sup> of 5 $\beta$ -androstane-3 $\beta$ -ol-17-one with *Penicillium* sp. ATCC 12,553, showed the compounds to be identical.

Incubation of 5 $\alpha$ -androstane-3,17-dione (24) produced two major products, 25 and 27 (Scheme III). The infrared spectrum of compound 25 showed a hydroxyl absorption and a broad carbonyl band. Mass spectral analysis gave a molecular weight of 304, which agreed with the elemental analysis of 25 for  $C_{19}H_{28}O_3$ , indicating that the starting dione had gained one oxygen atom. Oxidation of 25 gave a trione identical with 5 $\alpha$ -androstane-3,6,17-trione (26). The nmr spectrum of 25 confirmed the presence of only one hydroxyl group and allowed assignment of a  $\beta$  configuration to the C-6 hydroxyl group. In the 5 $\alpha$  series of steroids, a 6 $\alpha$ -hydroxyl substituent, being equatorial, has only a minor effect on the chemical shifts of the C-18 and C-19 methyl signals relative to the chemical shifts of the same methyls in the unsubstituted steroid; the C-19 methyl signal is shifted upfield 0.5 cps and the C-18 methyl signal is shifted downfield 0.5 cps.<sup>16</sup> However, the 1,3-diaxial relationship of a 6 $\beta$ -hydroxyl group and the C-19 methyl group results in a much larger shift for the C-19 methyl signal; the C-19 and C-18 methyl signals are shifted downfield by 13.5 and 2.5 cps, respectively.<sup>16</sup> The nmr spectrum of 25 exhibited the C-19 and C-18 methyl signals at 75 and 56 cps, respectively, *i.e.*, 12.5 and 2.5 cps downfield from the corresponding signals for 5 $\alpha$ -androstane-3,17-dione, measured under the same conditions.

The specific rotation of 25 provided further proof for assignment of a  $\beta$  configuration to the hydroxyl at C-6. Assuming a molecular rotation contribution ( $\Delta M_D$ ) of +55 for a 6 $\alpha$ -hydroxyl substituent<sup>17</sup> in 5 $\alpha$ -androstane-3,17-dione, a specific rotation of +112° would be expected for 5 $\alpha$ -androstane-6 $\alpha$ -ol-3,17-dione. However, assuming a  $\Delta M_D$  of -50 for a 6 $\beta$ -hydroxyl substituent,<sup>17</sup> a specific rotation of +80° would be expected for 5 $\alpha$ -androstane-6 $\beta$ -ol-3,17-dione, which is in good agreement with the measured specific rotation of +89° for compound 25.

Compound 27, the second metabolite from 24, was identified as follows. Mass spectral and elemental analysis indicated a molecular formula of  $C_{19}H_{28}O_4$ . The infrared spectrum of 27 showed an hydroxyl absorption at 3400 and a single carbonyl absorption at 1705  $cm^{-1}$ , suggesting that the C-17 ketone had undergone lactonization. This conclusion was supported when the infrared spectrum of 27 was found to be identical with that of 11 $\beta$ -hydroxy-5 $\alpha$ -dihydrotestolactone (11 $\beta$ -hydroxy-17 $\alpha$ -oxa-D-homo-5 $\alpha$ -androstane-3,17-dione). This compound had been obtained previously<sup>18</sup> by perfusion of testolactone through bovine adrenal glands. Confirmation of the structure of 27 came from the following microbial and chemical synthesis of an authentic sample. Although testolactone was the end product of incubation of progesterone with *A. tamarii*, when testolactone was incubated a low yield of 11 $\beta$ -hydroxytestolactone (28) was obtained. None of compound 28 was obtained after incubation of progesterone for 7 days. The structure proof of 28 rests upon comparison of it and its oxidation product (33) with authentic samples.<sup>19</sup> Hydrogenation of 28 gave a product which was identical with compound 27 obtained from 5 $\alpha$ -androstane-3,17-dione (24).

Having found a dihydro analogy to the conversion of androstenedione to testolactone, we were encouraged to extend the analogy to the dihydro derivatives of testosterone and progesterone, and thus achieve another case of sequential side-chain cleavage with D-ring lactone formation.

Incubation of androstanolone (29) gave a crude product consisting of one minor and two major components. The minor component was identified as 5 $\alpha$ -androstane-3,17-dione (24). One of the major components gave an infrared spectrum identical with that of 5 $\alpha$ -androstane-11 $\beta$ ,17 $\beta$ -diol-3-one (30). This compound has been obtained previously<sup>20</sup> by C. Meystre, but the work is as yet not published. As might be expected after finding dione 24, the second major component of the transformation mixture was 11 $\beta$ -hydroxy-5 $\alpha$ -dihydrotestolactone (27).

Incubation of 5 $\alpha$ -pregnane-3,20-dione (31) gave three metabolites. Compounds 24 and 27 were shown to be minor products of the transformation. The infrared spectrum of the major product (32) exhibited

(17) L. F. Fieser and M. Fieser, "Steroids," Reinhold Publishing Corp., New York, N. Y., 1959, p 179.

(18) G. M. Picha, U. S. Patent 2,755,289 (July 17, 1956); *Chem. Abstr.*, **51**, 2078 (1956).

(19) The only report in the literature of 11 $\alpha$ -hydroxytestolactone is ref 10; however, no physical properties are given. Dr. P. A. Diassi, Squibb Institute for Medical Research, New Brunswick, N. J., reports the compound to have mp 216°,  $[\alpha]_D^{25} +33^\circ$  (personal communication).

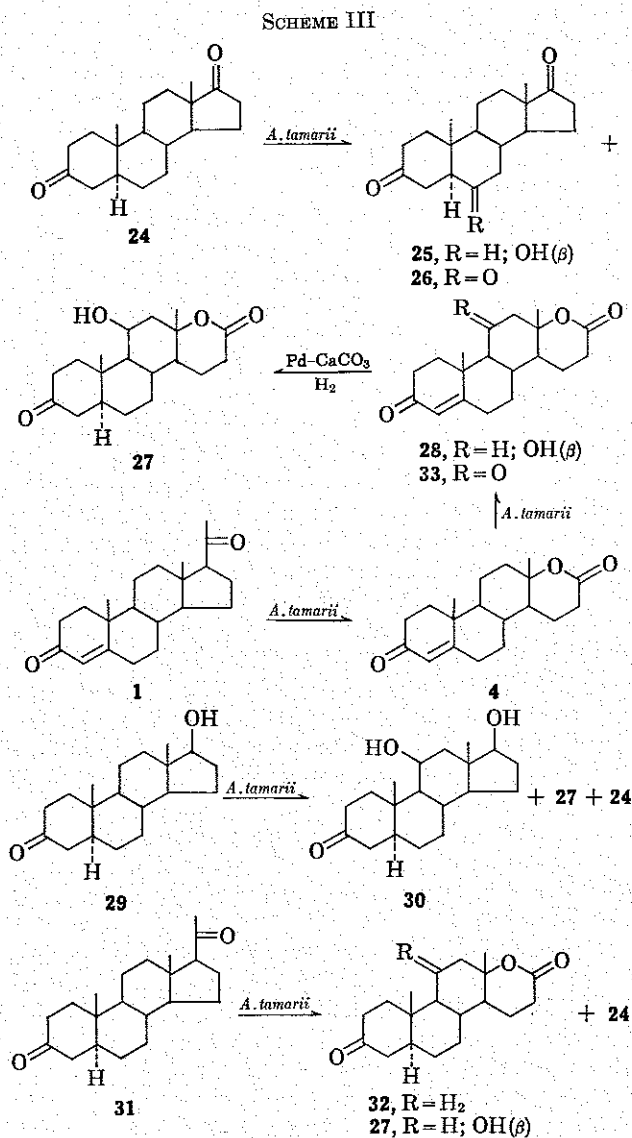
(20) Personal communication from Dr. R. Neher, Ciba Ltd., Basle, Switzerland, to whom we are also indebted for a sample of the compound.

(14) P. Talalay, *Ann. Rev. Biochem.*, **34**, 347 (1965).

(15) S. Noguchi and D. K. Fukushima, *J. Org. Chem.*, **30**, 3552 (1965).

(16) N. S. Bhacca and D. H. Williams, "Applications of Nmr Spectroscopy in Organic Chemistry," Holden-Day, Inc., San Francisco, Calif., 1964, p 19.

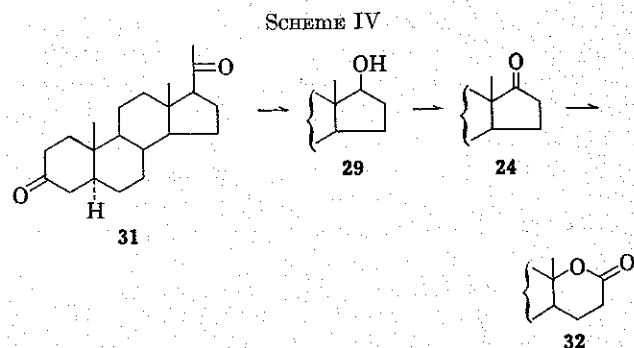
no hydroxyl absorption, but did contain a single carbonyl absorption at the same frequency as the carbonyl absorption of lactone 27. (See Scheme III).



Hydrogenation<sup>2</sup> of testolactone gives a mixture of compounds, one of which (5 $\alpha$ -dihydrotestolactone) was identical with compound 32. The occurrence of compound 32 again indicates that D-ring lactonization occurs prior to C-11 hydroxylation. This was substantiated when incubation of either 5 $\alpha$ -androstane-11 $\beta$ -ol-3,17-dione or diol 30 resulted only in recovery of starting material, indicating neither to be a precursor of lactone 27. However, incubation of lactone 32 afforded a good yield of lactone 27.

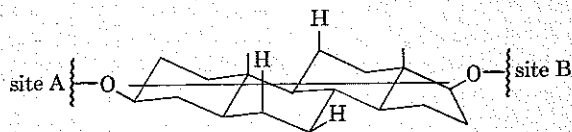
Conversion of 5 $\alpha$ -dihydroprogesterone (31) to 5 $\alpha$ -dihydrotestolactone (32) and conversion of both 5 $\alpha$ -dihydrotestosterone (29) and 5 $\alpha$ -dihydroandrostenedione (24) to a 5 $\alpha$ -dihydrotestolactone derivative imply the conversion shown in Scheme IV, which is analogous with the progesterone to testolactone conversion shown in Scheme I.

It is interesting to speculate on the substrate specificity shown by *A. tamaritii* toward hydroxylation of compounds 10, 21, and 24 in the C-11 $\beta$ , C-7 $\beta$ , and C-6 $\beta$  positions, respectively. Studies<sup>21,22</sup> have shown that



enzymatic steroid hydroxylations proceed by a mechanism in which there is a direct replacement of the hydrogen in the position to be hydroxylated, and<sup>23,24</sup> that the oxygen of the hydroxyl group introduced into the steroid originates from atmospheric oxygen and not from water.

It may be that each of the three substrates is hydroxylated by a different hydroxylase enzyme (either constitutive or produced by adaptation) depending upon the suitability of the substrate for attachment or attack by one of the three enzymes.<sup>25</sup> However, examination of Dreiding models of the steroid substrates and products suggests other possible explanations of the microbial hydroxylation specificity described above and for some cases reported in the literature. In view of the well-demonstrated stereoselectivity of microbial transformations it is probable that the steroid substrate is at some time bound to the surface of the enzyme causing the transformation, and that the points of binding having a fixed spatial relationship to the site of hydroxylase activity on the enzyme. In the three substrates mentioned above, and, in general, in most steroidal substrates, the most likely sites for binding the steroid to the enzyme surface are the oxygen functions at C-3 and C-17 (or C-20). Assuming these points of attachment, it is informative to note the spatial positions of the  $\beta$  hydrogens at C-6, C-7, and C-11 in relation to a line joining the oxygen functions at C-3 and C-17 as shown. The structure depicted is in-



tended to be only a model and not any particular steroid. At least two mechanisms (other than the multienzyme mechanism presented above) now become possible which could account for hydroxylation in the three different positions with only one enzyme. (1) The different conformation of each of the substrates causes the substrate to be orientated differently along the C-3-C-17 line, relative to the enzyme surface, such that either the C-6 $\beta$ , C-7 $\beta$ , or C-11 $\beta$ -hydrogen is nearest to the site of hydroxylase activity on the enzyme surface. (2) The steroid substrates may bind to the surface of the enzyme in more than one of the four possible orientations shown (I-IV); in each

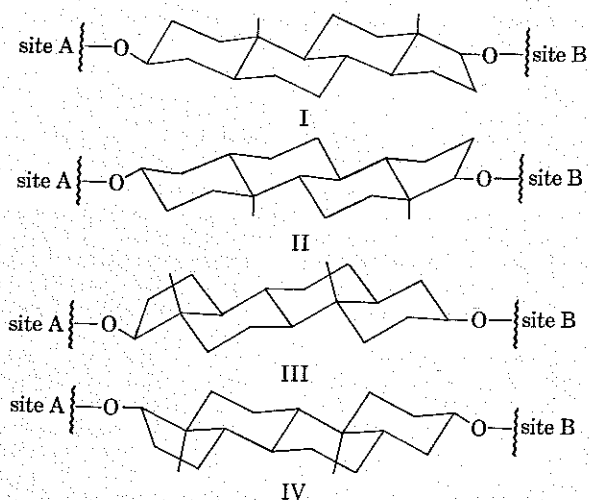
(21) E. J. Corey, G. A. Gregoriou, and D. H. Peterson, *J. Am. Chem. Soc.*, **80**, 2338 (1958).

(22) M. Hayano, et al., *ibid.*, **80**, 2336 (1958).

(23) M. Hayano, et al., *Arch. Biochem. Biophys.*, **59**, 529 (1955).

(24) M. Hayano, *Biochim. Biophys. Acta*, **21**, 380 (1956).

(25) S. H. Eppstein, et al., *J. Am. Chem. Soc.*, **75**, 408 (1953).



orientation a different hydrogen is nearest to the site of hydroxylase activity. Consequently the position of the  $11\beta$  hydrogen in I, relative to the site of  $11\beta$ -hydroxylase activity on the enzyme, becomes the midpoint between the C-6 and C-7  $\beta$ -hydrogens in III. This may account for the  $\beta$ -hydroxylations at C-11, C-6, and C-7 by *A. tamarii* as described above.

Many other cases have been reported of one organism having the ability to hydroxylate steroids in two different positions which tend to substantiate mechanism 2. *Penicillium* sp. ATCC 12,556 hydroxylates  $5\beta$ -androstane- $3\alpha$ -ol-17-one in the  $7\beta$  position. However, incubation of  $5\alpha$ -androstane- $3\alpha$ -ol-17-one with the same organism affords  $12\beta$  hydroxylation, whereas  $5\alpha$ -androstane- $3\beta$ -ol-17-one was hydroxylated in the  $1\alpha$  position.<sup>15</sup> It is interesting that the position of the  $1\alpha$  hydrogen in I, relative to the site of  $1\alpha$ -hydroxylase activity on the enzyme, is occupied by the  $12\beta$  hydrogen in IV.

Numerous organisms have been reported<sup>26</sup> which hydroxylate steroids in both the C- $6\beta$  and C- $11\alpha$  positions to give dihydroxy steroids. Studies<sup>27,28</sup> have shown that both hydroxylations do not occur simultaneously. Again, in going from I to IV the  $6\beta$  hydrogen is the hydrogen nearest to the space previously occupied by the  $11\alpha$  hydrogen.

It has been reported<sup>29</sup> that some organisms which give  $11\beta$  hydroxylation also give  $14\alpha$  hydroxylation, whereas organisms which give  $11\alpha$  hydroxylation, in general, do not give  $14\alpha$  hydroxylation. These observations could also be explained by mechanism 2. In going from I to II, the  $14\alpha$  hydrogen occupies the position previously occupied by the  $11\beta$  hydrogen.

Mechanism 2 may also explain the fact that several organisms, such as *A. tamarii*, are capable of hydroxylating the same substrate in either the C- $11\alpha$  or C- $11\beta$  position; in going from I to IV the  $11\alpha$  hydrogen is the hydrogen nearest to the position previously occupied by the  $11\beta$  hydrogen. We realize that little concrete evidence has been presented to substantiate mechanisms 1 or 2 proposed above. It may well be that numerous mechanisms are operative in microbial hydroxylations. Unfortunately, in many steroid trans-

formation studies only the major metabolites have been isolated and identified. Complete delineation of microbial hydroxylation mechanisms must await studies with pure hydroxylase enzymes.

### Experimental Section

Melting points were determined with a Thomas-Hoover melting point apparatus and are uncorrected. Analyses were performed by Mr. Carmine DiPietro of these laboratories. Infrared spectra were recorded with a Beckman IR-9 spectrometer, optical rotations were determined with a Bendix automatic polarimeter, ultraviolet spectra were obtained with a Cary 14 recording spectrometer, nmr spectra were determined with a Varian A-60 spectrometer using  $\text{CDCl}_3$  as solvent with TMS (0.00 cps) as internal standard, and mass spectra were taken on a CEC 21-110 instrument. Thin layer chromatograms were run on 275- $\mu$ -thick Merck silica gel G coated glass plates, with detection by iodine vapor. Fisher absorption alumina A-540 (activity III) was used for alumina column chromatography.

**General Methods of Fermentation.**—Flasks (250 ml) containing 100 ml of sterile solution<sup>30</sup> consisting of 50 g of glucose, 2.4 g of ammonium nitrate, 2 g of magnesium sulfate, 10 g of potassium dihydrogen phosphate, trace elements and 1000 ml of distilled water were inoculated with *A. tamarii* Kita QM 1223 and incubated at  $25^\circ$  for 96 hr on a rotary shaker. A solution of 50 mg of steroid dissolved in 0.4 ml of dimethylformamide was then added and the incubation was continued for another 96 hr, unless otherwise indicated. An average of 2 g of dry mycelium was obtained from each flask after the total 8 days. The flask contents were combined and the mycelium was filtered off and washed with chloroform. The aqueous filtrate was extracted by stirring for 24 hr with one-half of its volume of chloroform. This chloroform extract was combined with the chloroform washings from the mycelium, dried over anhydrous magnesium sulfate, and concentrated to dryness under vacuum.

**Incubation of Androsterone.**—Androsterone (1 g) was incubated for 96 hr as described above. Removal of the chloroform and dimethylformamide from the crude extract gave 550 mg of partially crystalline material which was chromatographed on 35 g of alumina.

Elution with methanol-ether (1:99) gave 310 mg of diol 6 which crystallized upon removal of the solvent: mp  $198-200^\circ$ ,  $[\alpha]_D^{25} +95^\circ$  (c 0.2, EtOH) (lit.<sup>31</sup> mp  $197-198^\circ$ ,  $[\alpha]_D^{25} +96^\circ$ ). Anal. Calcd for  $\text{C}_{19}\text{H}_{30}\text{O}_3$ : C, 74.47; H, 9.87. Found: C, 74.19; H, 10.03.

The infrared spectrum of 6 was superimposable on that of authentic  $5\alpha$ -androstane- $3\alpha$ , $11\beta$ -diol-17-one.<sup>32</sup>

Elution with methanol-ether (3:97) gave 70 mg of impure material which was rechromatographed on 10 g of alumina. Elution with methanol-ether (2:98) gave 45 mg of metabolite 7 which crystallized upon removal of the solvent: mp  $214-216^\circ$ ;  $[\alpha]_D^{25} +11^\circ$  (c 0.3,  $\text{Me}_2\text{CO}$ );  $\nu_{\text{max}}^{\text{KBr}}$  3400, 1705,  $1040\text{ cm}^{-1}$ . The mass spectrum of 7 showed the parent ion at  $m/e$  306.2177. Anal. Calcd for  $\text{C}_{19}\text{H}_{30}\text{O}_3$ : C, 74.47; H, 9.87. Found: C, 74.15; H, 10.03.

**Oxidation of Compound 7.**—Chromic oxide (15 mg) in 2 ml of acetone was added to 30 mg of compound 7 in 1 ml of acetone. After stirring at room temperature for 6 hr the acetone was removed and the residue was partitioned between water and ether. The ether portion was washed and dried ( $\text{MgSO}_4$ ) and the ether was removed to give 22 mg of noncrystalline solid which was chromatographed on 10 g of alumina.

Elution with benzene-ether (1:1) gave 5 mg of trione 9 mp  $175-177^\circ$  alone and on admixture. The infrared spectrum of 9 was identical with that of authentic<sup>32</sup>  $5\alpha$ -androstane- $3,11,17$ -trione.

Elution with methanol-ether (1:99) gave 10 mg of compound 8, mp  $190-191^\circ$ . The infrared spectrum of 8 was identical with that of authentic  $5\alpha$ -androstane- $11\alpha$ -ol- $3,17$ -dione.<sup>33</sup>

(26) S. C. Prescott and C. G. Dunn, "Industrial Microbiology," McGraw-Hill Book Co., Inc., New York, N. Y., 1959, pp 753-757.

(27) S. H. Eppstein, et al., *Vitamins Hormones*, **14**, 398 (1959).

(28) E. L. Dulaney, E. O. Stapley, and C. Hlavac, *Mycologia*, **47**, 464 (1955).

(29) S. H. Eppstein, et al., *Vitamins Hormones*, **14**, 390 (1959).

(30) P. W. Brian, et al., *J. Exptl. Botany*, **12**, 1 (1961). Repeating our earlier<sup>12</sup> conversions with this medium gave results identical with those obtained with 3% malt extract medium.

(31) H. L. Mason and E. J. Kepler, *J. Biol. Chem.*, **161**, 235 (1965).

(32) Kindly provided from the Steroid Reference Collection, Medical Research Council, by Professor W. Klyne and Dr. D. Kirk.

(33) Kindly provided by Dr. A. Wettstein, Ciba Ltd., Basle, Switzerland.

**Incubation of Isoandrosterone.**—Incubation of isoandrosterone (500 mg) gave 210 mg of crude product whose thin layer chromatogram (methanol-ether 2:98) showed only one major spot of  $R_f$  identical with that of 5 $\alpha$ -androstane-3 $\beta$ ,11 $\beta$ -diol-3-one. Chromatography of the crude product on 10 g of alumina using methanol-ether (1:99) as eluent gave 150 mg of compound 11. Recrystallization from methanol-ether gave an analytical sample, mp 228–230° (lit.<sup>34</sup> mp 228–231°). The infrared spectrum of compound 11 was identical with that of an authentic sample.<sup>32</sup>

**Incubation of 1,4-Androstadiene-3,17-dione.**—Incubation of 1,4-androstadiene-3,17-dione with *A. tamaris* for 96 hr as previously described gave 820 mg of material whose thin layer chromatogram (methanol-ether, 1:10) showed four components with  $R_f$  0.60, 0.65, 0.80, and 0.75, the latter component being relatively minor. Incubation of the same substrate for 48 hr gave a crude product whose thin layer chromatogram now showed the component of  $R_f$  0.60 to be present in a trace amount, relative to the components of  $R_f$  values 0.65, 0.75, and 0.80. The crude product from the 96-hr conversion was chromatographed on 20 g of alumina.

Elution with ether gave 210 mg of compound 15,  $R_f$  0.80. Recrystallization from ether gave an analytical sample, mp 178–180° (lit.<sup>35</sup> mp 176–179°). *Anal.* Calcd for  $C_{19}H_{24}O_3$ : C, 75.97; H, 8.05. Found: C, 75.70; H, 8.22. The infrared spectrum of 15 was identical with that of an authentic sample.<sup>36</sup>

Further elution with ether gave 311 mg of crystalline metabolite 13;  $R_f$  0.75, mp 165–166°. The infrared spectrum of 13 was identical with the spectrum of a commercial sample of 1,4-androstadien-17 $\beta$ -ol-3-one.

Elution with methanol-ether (1:99) gave 14 mg of compound 14;  $R_f$  0.65, mp 210–212° (lit.<sup>37</sup> mp 217°). *Anal.* Calcd for  $C_{19}H_{24}O_3$ : C, 75.97; H, 8.06. Found: C, 75.90; H, 8.01.

The infrared spectrum of 14 was identical with that of an authentic sample<sup>38</sup> of 1,4-androstadiene-11 $\beta$ ,17 $\beta$ -diol-3-one.

Elution with methanol-ether (3:97) gave 183 mg of compound 16,  $R_f$  0.60. Recrystallization from ether gave an analytical sample: mp 210–212°,  $[\alpha]^{25}_D + 85^\circ$  (c 0.1, Me<sub>2</sub>CO) (lit.<sup>39</sup> mp 212–214°,  $[\alpha]^{25}_D + 86.5^\circ$ ).

**Oxidation of 16 to Trione 17.**—To a solution of 10 mg of CrO<sub>3</sub> in 2 ml of acetone was added 20 mg of 16 in 1 ml of acetone. After stirring at room temperature for 12 hr the acetone was removed under vacuum and the residue was partitioned between ether and water. The ether portion was worked up as usual to give 13 mg of trione 17, mp 193–195° (lit.<sup>40</sup> mp 195–196°). *Anal.* Calcd for  $C_{19}H_{22}O_3$ : C, 76.48; H, 7.43. Found: C, 76.62; H, 7.31. The infrared spectrum of trione 17 was identical with that of a commercial sample of 1,4-androstadiene-3,11,17-trione.

The crude extract from the 48-hr conversion was chromatographed under the conditions described above. The same four products were isolated; however, compound 13 constituted only 10% of the crude product.

**Incubation of 1,4-Androstadien-17 $\beta$ -ol-3-one.**—Incubation of 1,4-androstadien-17 $\beta$ -ol-3-one (100 mg) gave 77 mg of crude product whose thin layer chromatogram showed spots with  $R_f$  0.75 and 0.65, but none of  $R_f$  0.80. The crude product was chromatographed on 10 g of alumina.

Elution with ether gave 18 mg of crystalline material,  $R_f$  0.75, identical with compound 13 described above.

Elution with methanol-ether (1:99) gave 45 mg of compound 14,  $R_f$  0.65, identical with 1,4-androstadiene-11 $\beta$ ,17 $\beta$ -diol-3-one described above. None of compound 15 was isolated.

**Incubation of 1,4-Androstadiene-11 $\beta$ ,17 $\beta$ -diol-3-one.**—Incubation of 1,4-androstadiene-11 $\beta$ ,17 $\beta$ -diol-3-one (100 mg) for 96 hr gave, after concentration of the chloroform extract, a crystalline product whose infrared spectrum was identical with that of starting diol 14. The thin layer chromatogram of the crude transformation product showed no spot with  $R_f$  value comparable with the  $R_f$  value of compound 15.

**Incubation of 1,4-Androstadiene-3,11,17-trione.**—Incubation of 1,4-androstadiene-3,11,17-trione (500 mg) gave 208 mg of 18. Addition of methanol-ether gave crystals: mp 230–232°;  $\nu^{KBr}_{max}$  3420, 1700, 1655, 1610 and 1590 cm<sup>-1</sup>;  $[\alpha]^{25}_D + 158^\circ$  (c 0.1, Me<sub>2</sub>CO) (lit.<sup>41</sup> mp 233.5–234.5°,  $[\alpha]^{25}_D + 169^\circ$ ). *Anal.* Calcd for  $C_{19}H_{24}O_3$ : C, 75.97; H, 8.05. Found: C, 76.20; H, 8.11.

A thin layer chromatogram of the crude transformation product showed no spots corresponding to 1,4-androstadien-11 $\alpha$ -ol-3,17-dione, or the corresponding 11 $\beta$ -hydroxydione.

**Incubation of 4-Androstene-3,11,17-trione.**—Addition of ether to the crude transformation product (370 mg) obtained from incubation of 4-androstene-3,11,17-trione (500 mg) gave 230 mg of crystalline compound 20: mp 180–181°;  $[\alpha]^{25}_D + 178^\circ$  (c 0.1, Me<sub>2</sub>CO);  $\nu^{KBr}_{max}$  3420, 1700, 1660, 1610 cm<sup>-1</sup> (lit.<sup>42</sup> mp 177–180°,  $[\alpha]^{25}_D + 182.4^\circ$ ). *Anal.* Calcd for  $C_{19}H_{26}O_3$ : C, 75.46; H, 8.67. Found: C, 75.61; H, 8.75. The residue from the crystallization was chromatographed on 10 g of alumina.

Elution with ether-benzene (1:1) gave 38 mg of starting trione, mp 215–217°.

Elution with ether gave 42 mg of compound 20 identical with that described above. A thin layer chromatogram of the crude transformation product showed no trace of either the 11 $\alpha$ - or 11 $\beta$ -hydroxy derivative of 4-androstene-3,17-dione.

**Incubation of 5 $\beta$ -Androstane-3,17-dione.**—Incubation of 5 $\beta$ -androstane-3,17-dione (500 mg) gave 360 mg of crude transformation product. A thin layer chromatogram (ethyl acetate) showed several trace components, but only one major component. The crude extract was chromatographed on 25 g of alumina.

Elution with ether-benzene (1:10) gave 52 mg of starting dione, mp 131–132°.

Elution with methanol-ether (1:99) gave 240 mg of compound 22. Crystallization from ethyl acetate gave an analytical sample: mp 170–172°,  $[\alpha]^{25}_D + 136^\circ$  (c 0.27, Me<sub>2</sub>CO) [lit.<sup>43</sup> mp 176°,  $[\alpha]^{25}_D + 145^\circ$  (CHCl<sub>3</sub>)]. *Anal.* Calcd for  $C_{19}H_{26}O_3$ : C, 74.96; H, 9.27. Found: C, 74.79; H, 9.40.

**Oxidation of 22 to Trione 23.**—To 20 mg of CrO<sub>3</sub> in 2 ml of acetone was added 50 mg of 22 in 3 ml of acetone. After stirring at room temperature for 12 hr the acetone was removed and the residue was partitioned between water and ether. The ether portion was worked up as usual to give 32 mg of trione 23 which crystallized upon addition of ethyl acetate and has mp 220–221°. The infrared spectrum of 23 was identical with that of authentic 5 $\beta$ -androstane-3,7,17-trione.<sup>43</sup> A thin layer chromatogram of the crude transformation product showed no component with the same  $R_f$  value as 5 $\beta$ -dihydrotestololactone.<sup>2</sup>

**Incubation of 5 $\alpha$ -Androstane-3,17-dione.**—Incubation of 5 $\alpha$ -androstane-3,17-dione (1 g) gave 720 mg of crude transformation product which was chromatographed on 25 g of alumina.

Elution with ether-benzene (1:10) gave 52 mg of crystalline starting dione 24.

Elution with ether gave 230 mg of 25 which crystallized upon addition of ethyl acetate: mp 215–216°;  $[\alpha]^{25}_D + 89^\circ$  (c 0.125, Me<sub>2</sub>CO);  $\nu^{KBr}_{max}$  3410, 1700–1720, 1420, 1180, and 1060 cm<sup>-1</sup>. The nmr spectrum showed singlets at  $\delta$  5.6 (3 H), 7.5 (3 H), and a multiplet at 2.33 (1 H) cps. The mass spectrum of 25 showed the parent ion at  $m/e$  304. *Anal.* Calcd for  $C_{19}H_{28}O_3$ : C, 74.96; H, 9.27. Found: C, 75.30; H, 9.31.

Elution with methanol-ether (3:97) gave 310 mg of compound 27. Recrystallization from ethyl acetate gave an analytical sample: mp 260–263° dec;  $[\alpha]^{25}_D - 12^\circ$  (c 0.1, Me<sub>2</sub>CO);  $\nu^{KBr}_{max}$  3400, 1705, 1220, 1090, and 1035 cm<sup>-1</sup>. The mass spectrum of 27 exhibited the parent ion at  $m/e$  320. *Anal.* Calcd for  $C_{19}H_{28}O_4$ : C, 71.22; H, 8.81. Found: C, 71.41; H, 8.86. The infrared spectrum of 27 was identical with that of authentic 11 $\beta$ -hydroxy-5 $\alpha$ -dihydrotestololactone.<sup>44</sup>

Elution with methanol-ether (1:10) gave 90 mg of crude solid, mp 160–165°, which has not been identified.

**Oxidation of 25 to Trione 26.**—To 25 mg of CrO<sub>3</sub> in 3 ml of acetone was added 50 mg of 25 in 3 ml of acetone. After stirring at room temperature for 12 hr the acetone was removed under vacuum and the residue was partitioned between water and ether.

(34) D. Kupfer, *Tetrahedron*, **15**, 193 (1961).

(35) W. Charney, *et al.*, *ibid.*, **18**, 591 (1962).

(36) Kindly furnished by Dr. H. L. Herzog, Schering Corp., Bloomfield, N. J.

(37) E. D. de Wit and J. de Visser, Dutch Patent 91,628 (July 15, 1959); *Chem. Abstr.*, **54**, 18604 (1959).

(38) Generously supplied by Dr. Katar Singh, Ayerst Laboratories, Montreal, Canada.

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(40) H. L. Herzog, *J. Am. Chem. Soc.*, **77**, 4781 (1955).

(41) R. C. Meeks, *et al.*, U. S. Patent 2,952,693 (Sept 13, 1960); *Chem. Abstr.*, **55**, 4880 (1960).

(42) H. L. Herzog, *J. Am. Chem. Soc.*, **75**, 266 (1953).

(43) Generously supplied by Dr. David K. Fukushima, Institute for Steroid Research, Montefiore Hospital and Medical Center, New York, N. Y.

(44) Kindly provided by Dr. Byron Riegel, G. D. Searle and Co., Chicago, Ill.

The ether portion was worked-up in the usual way to give 38 mg of trione 26. Two recrystallizations from ether gave crystals mp 194–196° (lit.<sup>45</sup> mp 193–195°). The infrared spectrum of 26 was identical with that of an authentic sample of 5 $\alpha$ -androstane-3,6,17-trione.<sup>43</sup>

**Incubation of Testolactone.**—Testolactone (1 g) was incubated for 96 hr under the conditions described above. Exhaustive chloroform extraction of the aqueous transformation medium gave 620 mg of crude material which was chromatographed on 30 g of alumina.

Elution with ether-benzene (1:1) gave 490 mg of starting lactone 4, mp 207–209° alone and on admixture.

Elution with methanol-ether (1:99) gave 50 mg of compound 28, which crystallized upon addition of ether: mp 263–265°;  $[\alpha]_D^{20} +56^\circ$  (*c* 1.0, CHCl<sub>3</sub>);  $\lambda_{max}^{alcohol}$  241 m $\mu$  ( $\epsilon$  15,700) (lit.<sup>18</sup> mp 263–267°,  $[\alpha]_D +41^\circ$ ). The mass spectrum of 28 gave the parent ion at *m/e* 318. *Anal.* Calcd for C<sub>19</sub>H<sub>26</sub>O<sub>4</sub>: C, 71.67; H, 8.23. Found: C, 71.78; H, 8.32. The infrared spectrum of 28 was identical with that of authentic 11 $\beta$ -hydroxytestolactone.<sup>44</sup>

**Oxidation of 28 to Keto Lactone 33.**—To 5 mg of CrO<sub>3</sub> in a ml of acetone was added 10 mg of 28 in 2 ml of acetone. After stirring for 10 hr the acetone was removed under vacuum and the residue was partitioned between water and ether. The ether portion was worked up in the usual way to give 5 mg of crystalline 33, mp 239–241° alone and on admixture. The infrared spectrum of 33 was identical with that of authentic 11-oxotestolactone.<sup>46</sup>

**Hydrogenation of 28 to Lactone 27.**—Compound 28 (30 mg) in 5 ml of absolute ethanol was hydrogenated at room temperature and atmospheric pressure in the presence of 5% palladium on calcium carbonate (15 mg). After removal of the catalyst by filtration, the ethanol was evaporated and the resulting residue was chromatographed on 5 g of alumina.

Elution with methanol-ether (1:99) gave 10 mg of crystalline material which was identical with compound 27 described above.

Elution with methanol-ether (3:97) gave 15 mg of crude mixture which was not further purified.

**Incubation of Androstanolone.**—Incubation of androstanolone (1.25 g) gave 810 mg of crude product which was chromatographed on 25 g of alumina.

Elution with benzene gave 179 mg of crystals whose *R<sub>f</sub>* value on tlc, specific rotation, and infrared spectrum were identical with those of 5 $\alpha$ -androstane-3,17-dione.

Elution with methanol-ether (1:99) gave 250 mg of dihydroxy ketone 30, which crystallized upon addition of ethyl acetate: mp 254–256° alone and on admixture,  $[\alpha]_D^{20} +41^\circ$  (*c* 1.0, CHCl<sub>3</sub>). *Anal.* Calcd for C<sub>19</sub>H<sub>30</sub>O<sub>3</sub>: C, 74.47; H, 9.87. Found: C, 74.70; H, 9.72. The infrared spectrum of 30 was super-

imposable on that of an authentic sample<sup>20</sup> of 5 $\alpha$ -androstane-11 $\beta$ ,17 $\beta$ -diol-3-one.

Elution with methanol-ether (3:97) gave 130 mg of compound 27. The melting point, elemental analysis, and infrared spectrum of this material were identical with those of compound 27 described above.

**Incubation of Diol 30.**—Incubation of diol 30 (150 mg) gave 96 mg of transformation product which crystallized upon removal of the chloroform and addition of ethyl acetate. This material was identical with starting diol 30. A thin layer chromatogram of the crude transformation product showed none of lactone 27.

**Incubation of 5 $\alpha$ -Pregnane-3,20-dione.**—Incubation of 5 $\alpha$ -pregnane-3,20-dione (1 g) gave a crude product (690 mg) which was chromatographed on 25 g of alumina.

Elution with benzene gave 40 mg of dione 24, mp 130–132° alone and on admixture.

Elution with ether-benzene (1:1) gave 290 mg of 32 which crystallized upon addition of ethyl acetate and hexane: mp 169–170°,  $[\alpha]_D^{25} -15^\circ$  (*c* 0.1, CHCl<sub>3</sub>) (lit.<sup>2</sup> mp 171–172°,  $[\alpha]_D^{25} -18^\circ$ ). *Anal.* Calcd for C<sub>19</sub>H<sub>28</sub>O<sub>3</sub>: C, 74.96; H, 9.27. Found: C, 75.05; H, 9.32.

Elution with ether-benzene (1:10) gave 165 mg of starting dione 31, mp 198–200°.

Elution with methanol-ether (1:99) gave 85 mg of material whose melting point and infrared spectrum were identical with those described above for 27.

**Incubation of 5 $\alpha$ -Dihydrotestolactone.**—Incubation of 5 $\alpha$ -dihydrotestolactone (200 mg) for 96 hr gave a crude mixture (110 mg) which was chromatographed on 10 g of alumina.

Elution with ether-benzene (1:1) gave 30 mg of material identical with lactone 32 described above.

Elution with methanol-ether (1:99) gave 56 mg of crystalline material identical with compound 27 described above.

**Incubation of 5 $\alpha$ -Androstan-11 $\beta$ -ol-3,17-dione.**—Incubation of 5 $\alpha$ -androstan-11 $\beta$ -ol-3,17-dione (250 mg) for 96 hr gave a crude extract (190 mg) which crystallized upon addition of ether. The thin layer chromatogram, melting point, and infrared spectrum of this material were identical with those of the starting hydroxydione.

**Registry No.**—5, 53-41-8; 6, 57-61-4; 7, 7801-12-9; 10, 570-27-4; 11, 514-17-0; 6, 897-06-3; 15, 898-84-0; 13, 846-84-0; 14, 7801-17-4; 16, 7801-18-5; 17, 7738-93-4; 18, 5885-11-0; 19, 382-45-6; 20, 564-35-2; 21, 1229-12-5; 22, 2260-02-8; 24, 846-46-8; 25, 7801-26-5; 27, 7801-27-6; 4, 4416-57-3; 28, 7801-29-8; 29, 521-18-6; 30, 7801-30-1; 31, 566-65-4; 32, 7801-32-3; 5 $\alpha$ -androstan-11 $\beta$ -ol-3,17-dione, 599-11-1.

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